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Rho GTPases are molecular switches that fluctuate between on and off states. When active, these proteins function to remodel the actin cytoskeleton by interacting with a number of downstream effector molecules. Recent studies have linked the activation of Rho GTPases with the acquisition of a metastatic phenotype in many types of cancers including inflammatory breast cancer (IBC). This proposal incorporates a rational approach to target these signaling proteins using small molecule inhibitors that would interfere with their ability to become activated by Rho family guanine nucleotide exchange factors (RhoGEFs). Our strategy incorporates both virtual, structure-based screening and a complementary high throughput drug screen to identify small molecule inhibitors that interfere with Rho GTPase activation and signal transduction. Any inhibitors identified through this research can serve as useful tools for studying Rho-mediated signal transduction cascades and may lead to the development of novel cancer therapeutics.

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Table of Contents

Annual Report 2004

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Breast Cancer Research Program Predoctoral Fellow
Proposal title: Rational Design of Rho Protein Inhibitors
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Introduction

Rho GTPases belong to the Ras superfamily of small (~21 kDa) monomeric guanine nucleotide-binding proteins. There are approximately 20 Rho subfamily members, the most characterized of which are RhoA, Rac1, and Cdc42. Like other guanine nucleotide binding proteins, Rho family members are molecular switches that fluctuate between active GTP-bound and inactive GDP-bound states. Rho GTPases are signaling molecules that can propagate signal transduction events initiated by extracellular stimuli. For example, activation of receptor tyrosine kinases, G-protein coupled receptors, or integrin receptors results in the activation of a Rho Guanine nucleotide Exchange Factor (RhoGEF), which facilitates the GTP-loading of specific Rho GTPases via the catalytic Dbl-homology (DH) domain [for reviews, see 1-5]. Once GTP-bound, Rho GTPases adopt an active conformation and are free to interact with downstream effector molecules. Rho GTPases are mediators of actin cytoskeletal remodeling and have been linked with the acquisition of a metastatic and invasive phenotype in several cancer types [7-19]. Recently, numerous reports have suggested that Rho GTPase activation is an integral step during the invasion and metastasis process of a wide variety of cancers including inflammatory breast cancer (IBC) [7-19]. Consequently, a recent issue of the journal *Breast Cancer Research and Treatment* was devoted exclusively to reports describing the link between Rho family GTPases and breast cancer progression and dissemination [7-13]. This predoctoral fellowship encompasses a rational approach to target the oncogenic signaling properties of Rho GTPases in order to control aberrant signaling in breast cancer as well as other diseases. Our approach to discovering novel inhibitors of Rho protein signal transduction encompasses both structure-based and high-throughput strategies and includes biochemical and cellular methods for subsequent characterization of lead compounds.

Results

***In silico* docking:** In the original statement of work proposal, **Task 1** (months 1-12) was dedicated to virtual docking of a compound library using RhoGEF structures recently defined by our group. A large majority of the outlined experiments within the proposed **Task 1** have been accomplished and experiments outlined in **Task 2** (months 13-19) are currently underway. Instead of using the NCI (National Cancer Institute) compound library we have chosen to use a library consisting of commercially available compounds provided by Ryan Scientific, because this database is of considerably higher quality than the NCI database, more readily available, and more amendable to virtual docking. The data output from these docking studies yields (1) minimized energy functions for a protein-ligand interaction consisting of electrostatic and van der Waals interactions and (2) a three-dimensional representation of the ligand in the most favorable orientation. We have completed initial docking of the RhoGEF crystal structures of both Tiam1 (T-cell invasion and metastasis factor 1) and Dbs (Diffuse B-cell lymphoma's big sister) and have begun testing predicted inhibitors using an *in vitro* guanine nucleotide exchange assay (described below). Figure (**1**) illustrates some results obtained from *in silico* docking studies of Tiam1 (see below for docking methods) and computational analysis of the ligand-binding site. By analyzing the Tiam1 DH domain surface using computational methods, as well as mutational studies (see below), a solvent accessible region was identified and used as the query for docking studies. The biophysical properties of this region is described and pictured in figure (**1a.-d.**). Additionally, figure (**1e.**) shows an example of data output from a recent Tiam1

docking study and the corresponding pharmacophore interactions made by 5 of the highest scoring compounds. We have observed that high scoring compounds typically share similar chemical features that make favorable interactions with the protein. We are currently determining the activity of these and other potential inhibitors.

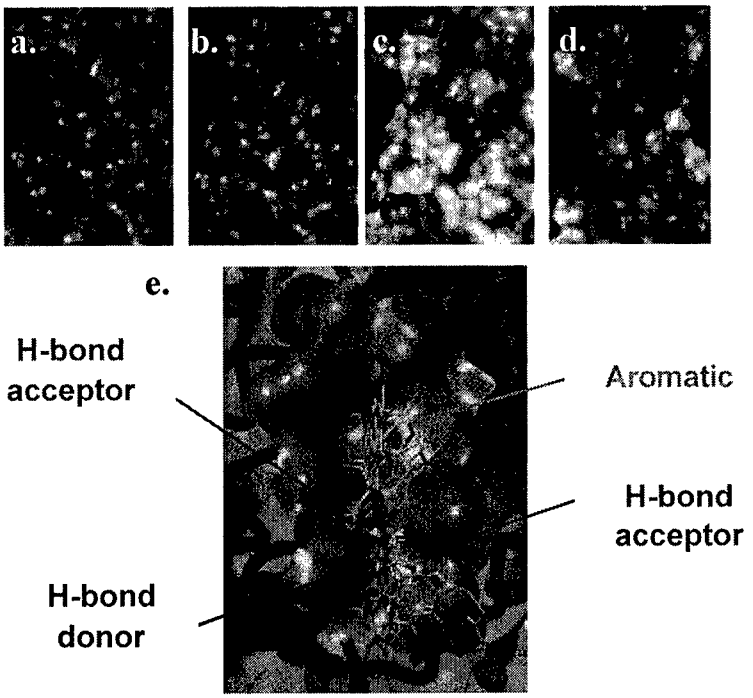

Fig. (1) Analysis of the ligand binding region used for *in silico* docking and computational analysis of predicted Tiam1 inhibitors. The solvent accessible surface of the Tiam1 DH domain was generated using MOLCAD (Tripos) and is color-coded to indicate (**a.**) hydrophobicity (brown), (**b.**) local curvature, (**c.**) H-bond donor regions (red), and (**d.**) H-bond acceptor regions (blue). This analysis was used to construct a query for docking purposes shown as a cyan surface in (**e.**) with other regions of the DH domain (alpha helices) shown as red tubes. Five of the highest scoring compounds predicted to bind Tiam1 are shown overlaid onto each other to illustrate common chemical groups that produce favorable interactions with the protein query including H-bond acceptors, H-bond donor, and aromatic groups.

Site-directed mutational analysis: Residues within the DH domain of Tiam1 were individually mutated to determine their contribution to Rac1 binding and activation (see methods below) in order to define key residues for small molecule targeting. The residues within Tiam1 known to make contacts to Rac1 in the crystal structure are outlined in figure (**2a.**) with the corresponding Rac1 interaction partner listed below each residue. Mutations made to the Tiam1 DH domain are listed at the bottom of figure (**2b.**) with experimentally determined exchange activity and binding affinity for Rac1 also shown in figure (**2b.**). The most deleterious mutations were also those that made extensive contacts to Rac1 in the crystal structure. For example, mutation of E1047, L1194, K1195, L1198, and N1232, which all displace over 50 $Å^2$, virtually eliminated any exchange activity or binding of Rac1. Of these, K1195 is within the closest proximity to the solvent accessible query site used for docking purposes. We aim to mimic the effects of these deleterious mutations using small molecules that bind near these critical residues.

High-throughput drug screening: Virtual drug screening by means of small molecule docking is an emerging technology that is in some ways superior to traditional methods for drug discovery, yet still retains some drawbacks [for a review, see 6]. For example, an advantage of virtual docking is the use of computational algorithms to take the place of expensive and time consuming high content screening. However, a disadvantage is the reliance on computational scoring algorithms for the characterization and ranking of potential inhibitors. For these reasons, we have also incorporated additional strategies for the identification of small molecule inhibitors of Rho GTPase activation, namely high throughput screening of small molecule libraries as described in the original proposal. This approach follows the methods outlined in **Task 2** and utilizes 384-well plated small molecule libraries. With the aid of our collaborators (Ross Stein Ph.D., Laboratory for Drug Discovery in Neurodegeneration, Harvard University), we are currently developing a 384-well high throughput format of our standard fluorescence-based guanine nucleotide exchange assay. We are currently using this assay to screen a

small molecule library of ~30,000 compounds for inhibitors of RhoA activation by the RhoGEF Dbs. While these studies are ongoing, our future plans are to publish details of the high throughput assay along with assay validation and statistical parameters. Furthermore, we will characterize any inhibitors identified using the screen using biochemical, structural, and cellular methods. As stated in the original proposal, this high throughput screen is a complimentary approach to the virtual docking studies. Funds provided by this predoctoral BCRP fellowship have allowed me to travel to Dr. Stein's lab in Boston MA, to be trained in high throughput screening technologies. This formal training has included assay development, conversion to automated format, and statistical techniques required for assay validation. More importantly, it has allowed me to see first hand the process behind anti-cancer drug discovery research from conception of an ideal drug target, to development of an assay and discovery of lead compounds.

Methods

***In silico* docking:** Solvent accessible sites on the surface of Tiam1 DH domain were predicted using SiteID option of Sybyl (Tripos). These sites were then evaluated using structural data and mutational analysis for their potential as a receptor query sites for docking purposes. The solvent accessible sites chosen for docking were shown to contain residues that contact the GTPase and are critical for nucleotide exchange shown in figure 2a.). A compound library was docked first using DOCK (UCSF) and the top 5,000 hits were then reanalyzed using using FlexX (Sybyl). The Tiam1 DH/PH – Rac1 pdb file (1FOE) was modified for docking purposes by deleting the GTPase chain, removing heteroatoms (ions, water), and changing selenomethione (MSE) to methionine in the pdb file. The receptor query was then used to construct a surface using the program Connolly (DOCK) and sph site points using the program Sphgen (DOCK). After adding hydrogens and assigning formal charges to the Tiam1 DH domain using Sybyl (Tripos), a scoring grid of was calculated using Grid (DOCK). Parameters for DOCK were standard flexible docking using a maximum of 100 configurations. The Ryan Scientific library of compounds was used as a virtual library for docking. The 2D coordinates of the compound library were converted into 3D mol2 files using the CONCORD standalone option in Sybyl. The top 5,000 hits obtained from DOCK were further analyzed using the FlexX (Tripos) docking algorithm and are currently being analyzed for activity.

Site-directed mutagenesis studies: Tiam1 point mutants were generated using a standard PCR-based mutagenesis protocol. Proteins were produced in E. Coli and purified using standard Ni-NTA affinity purification methods followed by size-exclusion chromatography. All purified proteins were first analyzed using circular dichroism spectral methods for proper folding, then tested for exchange activity using an *in vitro* guanine nucleotide exchange assay (described below) and tested for Rac1 binding using surface plasmon resonance. GST-fusion Rac1 protein was bound to an anti-GST surface (Biacore) and Tiam1 mutants were the analyte flowed over to analyze binding in a dose-dependent manner. A GST-only surface was used as a negative control for binding, and buffer-only was subtracted from all binding isotherms to eliminate background signal. The binding affinity for each Tiam1 mutant was then determined in triplicate by fitting each binding isotherm to a standard one site binding model.

Guanine nucleotide exchange assays: RhoGEFs catalyze the exchange of guanine nucleotide on Rho GTPases. In order to monitor activity of Tiam1, we performed a fluorescence-based guanine nucleotide exchange assay, as described in [20]. Proteins were constituted in 1X exchange buffer consisting of 10% glycerol (v/v), 20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM DTT, and 10 mM $MgCl_2$. Conditions for the cuvette-based fluorescence guanine nucleotide exchange assay consisted of 2 μM GTPase (Rac1), 500nM BODIPY-conjugated GTP, and 200-400 nM RhoGEF in 1X exchange buffer at total volume of 1,000 μL. Cuvettes were equilibrated at 20°C and stirred constantly. After ~500 sec of pre-incubation without RhoGEF, the appropriate amount of RhoGEF (Tiam1) was

manually pipetted into the reaction mixture. Fluorescence was measured in real time using a Perkin-Elmer LS-55 with lambda$_{ex}$ = 590 nm and lambda$_{em}$ = 620 nm.

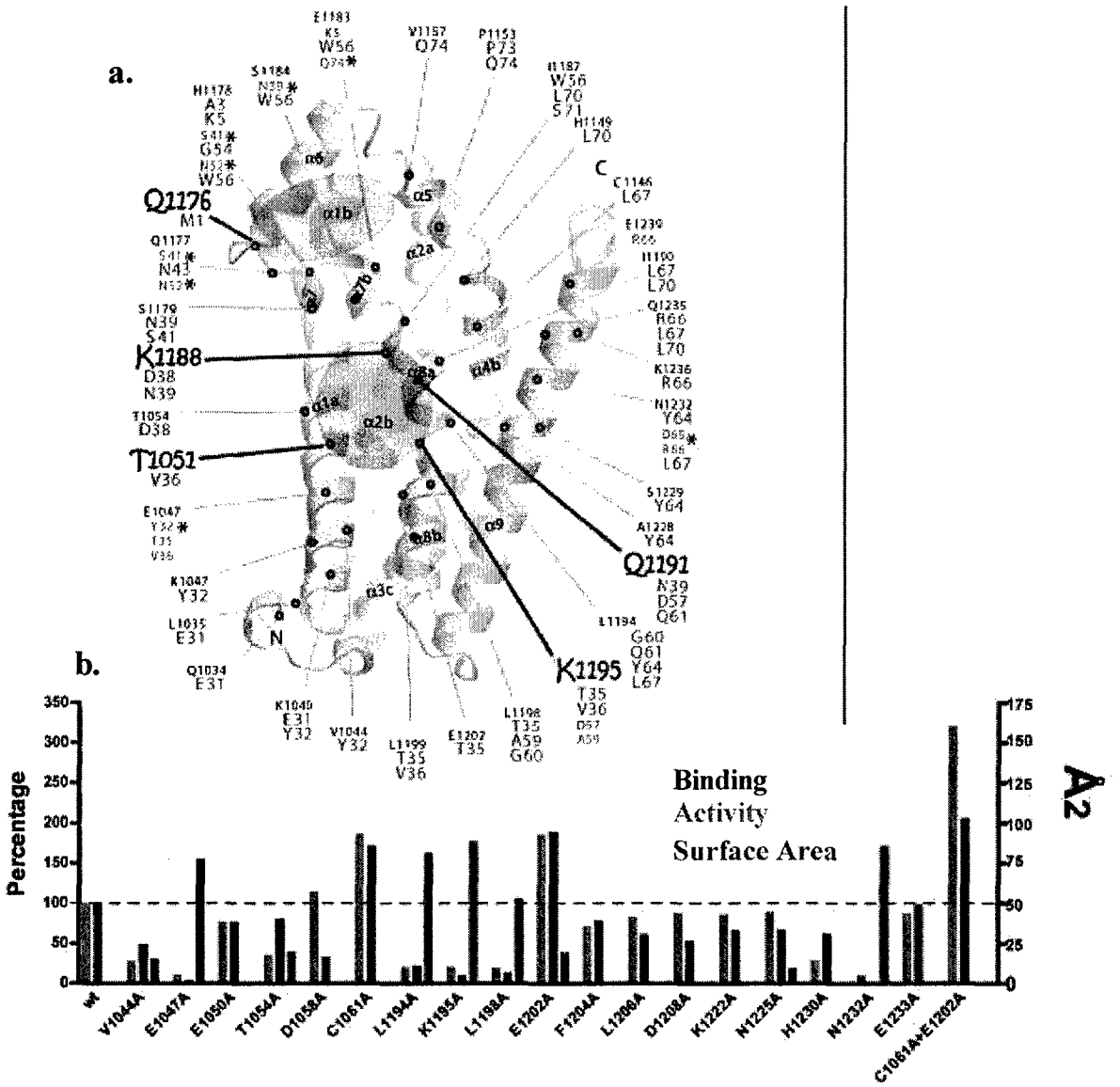

Fig. (2) Structural and mutational analysis of the Tiam1-Rac1 interaction reveals critical sites for activity. **a.** Secondary structure representation of the Tiam1 DH domain is shown in yellow with alpha helices labeled (modified from [21]). Residues of Tiam1 that make contacts to Rac1 are listed in black. Underneath each Tiam1 residue number is the corresponding Rac1 interacting partner residue(s). These listed Rac1 residues are color-coded to indicate the nature of the interaction made to Tiam1 as follows: Red, van der Waals; green, hydrogen bonds; blue, ionic bonds; *, non-ionic side chain interactions. Solvent accessible sites on the Tiam1 surface were predicted using SiteID (Tripos) and are shown as two colored circles; residues within these sites that make contacts to Rac1 are highlighted with red lines and large bold black text. These two sites comprise the ligand binding regions for *in silico* docking studies. **b.** Point mutations made in Tiam1 are listed at bottom with the corresponding surface area displacement (scaled on right, Å^2) upon binding Rac1, as determined from the crystal structure, shown in red. The activity of each Tiam1 mutant was tested using an *in vitro* guanine nucleotide exchange assay, shown in green as the percent activity compared to wild type (wt). Binding affinities for each Tiam1 point mutant were analyzed using surface plasmon resonance (Biacore) and are also shown as percentages of wt Tiam1 in blue. A black dashed line is shown for 100% activity or binding as a reference. The most deleterious Tiam1mutations correlate well with residues that make extensive contacts to Rac1. Additionally, two independent activating point mutations were identified (C1051A, E1202A) which are currently being investigated.

Key Research Accomplishments

Virtual drug discovery

- Computational analysis of solvent accessible sites at the GTPase/RhoGEF interface of Rac1/Tiam1 and RhoA/Dbs
- Mutational analysis of Tiam1 residues required for binding and activating Rac1
- Reformatting of the Ryan Scientific small molecule library (>300,000 compounds) for virtual screening purposes; conversion from sdf format to mol2 3-D format
- Virtual screening of both Tiam1 and Dbs binding surface utilizing the algorithms DOCK and FlexX
- Analysis of virtual compound hits, initiation of testing compounds for activity using a functional guanine nucleotide exchange assay, as well as binding studies

High throughput assay development (ongoing)

- Purification of large quantities of proteins (100-300 mg each) for use in high throughput screening and secondary studies, including Dbs, Tiam1, Sos1, RhoA, Cdc42, Rac1, and H-Ras
- Conversion of a kinetic fluorescence-based guanine nucleotide exchange assay to high throughput, automated 384-well format for drug screening purposes
- Initiation of a new automated 384-well formatted drug screen consisting of ~30,000 compounds through a collaboration with Dr. Ross Stein, Laboratory for Drug Discovery in Neurodegeneration, Harvard University

Reportable Outcomes

N/A

Conclusions

In conclusion, recent studies have further linked Rho GTPase activation to cancer progression and acquisition of a metastatic phenotype in many types of cancers including breast cancers [7-19]. This proposal incorporates a rational approach to target these signaling proteins using small molecule inhibitors that would interfere with their ability to become activated by RhoGEFs. To this end, we have initiated both a virtual drug screening strategy and a high throughput approach with the aim of identifying small molecule inhibitors that may be characterized using biochemical, as well as cellular methods.

I have received formal training in several areas that will support my future as a cancer researcher. This has included training in computational techniques including three dimensional structure manipulation, virtual small molecule docking, protein purification techniques, high-throughput assay development, and am currently being trained in X-ray crystallographic techniques for structure determination. This training program has consisted of formal training either individually from experts in the field either at UNC and Harvard University or in a group setting in the form of course work and workshops. For example, I have received bioinformatics training from the director of the UNC structural bioinformatics core facility (Brenda Temple, Ph.D.) and have also been mentored by experts in high-throughput assay development at the Laboratory for Drug Development (Ross Stein, Ph.D. and Li-An Yeh, Ph.D.). Additionally, I am currently receiving X-ray crystallographic training from the director of the X-ray core facility at UNC (Laurie Betts, Ph.D.). This training program will be invaluable to my development as a cancer researcher and will also aid the progression of this project.

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Appendices
N/A